

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

## CORRECTED VERSION

(19) World Intellectual Property  
Organization  
International Bureau



(43) International Publication Date  
18 March 2004 (18.03.2004)

PCT

(10) International Publication Number  
WO 2004/022764 A3

- (51) International Patent Classification<sup>7</sup>: C12P 13/04, 7/04, 41/00, 12/22, 13/06, C12N 15/53, 9/04
- (21) International Application Number:  
PCT/EP2003/008631
- (22) International Filing Date: 5 August 2003 (05.08.2003)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:  
102 40 603.0 3 September 2002 (03.09.2002) DE
- (71) Applicant (*for all designated States except US*): DE-GUSSA AG [DE/DE]; Bennigsenplatz 1, 40474 Düsseldorf (DE).
- (72) Inventors; and
- (75) Inventors/Applicants (*for US only*): NÁAMNIEH, Shukrallah [IL/DE]; Stippelburggasse.20, 40221 Düsseldorf (DE). HUMMEL, Werner [DE/DE]; Claudiusstrasse 11, 52445 Titz (DE). GRÖGER, Harald [DE/DE]; Akademiestrasse 31, 63450 Hanau (DE).
- (74) Common Representative: DEGUSSA AG; Intellectual Property Management, PATENTE und MARKEN, Standort Hanau, Postfach 13 45, 63403 Hanau (DE).
- (81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.
- (84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).
- Published:  
— with international search report
- (88) Date of publication of the international search report:  
21 October 2004
- (48) Date of publication of this corrected version:  
14 April 2005
- (15) Information about Correction:  
see PCT Gazette No. 15/2005 of 14 April 2005, Section II
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

(54) Title: USE OF MALATE DEHYDROGENASE FOR NADH REGENERATION

(57) Abstract: The present invention relates to a process for the preparation of enantiomerically enriched organic compounds. In particular, the present invention relates to an enzymatically operating process, in which, in a coupled enzymatic reaction system, NAD(P)H is consumed by one enzyme for the preparation of the organic compound and the NAD(P)H is simultaneously regenerated by a second enzyme system. A reaction system which operates according to the invention in this manner and an advantageous whole cell catalyst are also proposed.

1. The first step in the process is to identify the problem or issue that needs to be addressed. This involves gathering information and understanding the context of the problem.

• *Journal of the American Medical Association*, 1997; 277: 1033-1037

... ..

16. *Journal of the American Medical Association*, 1990; 263: 1033-1036.

1987-1988 1989-1990 1991-1992 1993-1994 1995-1996 1997-1998 1999-2000 2001-2002 2003-2004 2005-2006 2007-2008 2009-2010 2011-2012 2013-2014 2015-2016 2017-2018 2019-2020 2021-2022 2023-2024 2025-2026 2027-2028 2029-2030 2031-2032 2033-2034 2035-2036 2037-2038 2039-2040 2041-2042 2043-2044 2045-2046 2047-2048 2049-2050 2051-2052 2053-2054 2055-2056 2057-2058 2059-2060 2061-2062 2063-2064 2065-2066 2067-2068 2069-2070 2071-2072 2073-2074 2075-2076 2077-2078 2079-2080 2081-2082 2083-2084 2085-2086 2087-2088 2089-2090 2091-2092 2093-2094 2095-2096 2097-2098 2099-2100 2101-2102 2103-2104 2105-2106 2107-2108 2109-2110 2111-2112 2113-2114 2115-2116 2117-2118 2119-2120 2121-2122 2123-2124 2125-2126 2127-2128 2129-2130 2131-2132 2133-2134 2135-2136 2137-2138 2139-2140 2141-2142 2143-2144 2145-2146 2147-2148 2149-2150 2151-2152 2153-2154 2155-2156 2157-2158 2159-2160 2161-2162 2163-2164 2165-2166 2167-2168 2169-2170 2171-2172 2173-2174 2175-2176 2177-2178 2179-2180 2181-2182 2183-2184 2185-2186 2187-2188 2189-2190 2191-2192 2193-2194 2195-2196 2197-2198 2199-2200 2201-2202 2203-2204 2205-2206 2207-2208 2209-2210 2211-2212 2213-2214 2215-2216 2217-2218 2219-2220 2221-2222 2223-2224 2225-2226 2227-2228 2229-2230 2231-2232 2233-2234 2235-2236 2237-2238 2239-2240 2241-2242 2243-2244 2245-2246 2247-2248 2249-2250 2251-2252 2253-2254 2255-2256 2257-2258 2259-2260 2261-2262 2263-2264 2265-2266 2267-2268 2269-2270 2271-2272 2273-2274 2275-2276 2277-2278 2279-2280 2281-2282 2283-2284 2285-2286 2287-2288 2289-2290 2291-2292 2293-2294 2295-2296 2297-2298 2299-2300 2301-2302 2303-2304 2305-2306 2307-2308 2309-2310 2311-2312 2313-2314 2315-2316 2317-2318 2319-2320 2321-2322 2323-2324 2325-2326 2327-2328 2329-2330 2331-2332 2333-2334 2335-2336 2337-2338 2339-2340 2341-2342 2343-2344 2345-2346 2347-2348 2349-2350 2351-2352 2353-2354 2355-2356 2357-2358 2359-2360 2361-2362 2363-2364 2365-2366 2367-2368 2369-2370 2371-2372 2373-2374 2375-2376 2377-2378 2379-2380 2381-2382 2383-2384 2385-2386 2387-2388 2389-2390 2391-2392 2393-2394 2395-2396 2397-2398 2399-2400 2401-2402 2403-2404 2405-2406 2407-2408 2409-2410 2411-2412 2413-2414 2415-2416 2417-2418 2419-2420 2421-2422 2423-2424 2425-2426 2427-2428 2429-2430 2431-2432 2433-2434 2435-2436 2437-2438 2439-2440 2441-2442 2443-2444 2445-2446 2447-2448 2449-2450 2451-2452 2453-2454 2455-2456 2457-2458 2459-2460 2461-2462 2463-2464 2465-2466 2467-2468 2469-2470 2471-2472 2473-2474 2475-2476 2477-2478 2479-2480 2481-2482 2483-2484 2485-2486 2487-2488 2489-2490 2491-2492 2493-2494 2495-2496 2497-2498 2499-2500 2501-2502 2503-2504 2505-2506 2507-2508 2509-2510 2511-2512 2513-2514 2515-2516 2517-2518 2519-2520 2521-2522 2523-2524 2525-2526 2527-2528 2529-2530 2531-2532 2533-2534 2535-2536 2537-2538 2539-2540 2541-2542 2543-2544 2545-2546 2547-2548 2549-2550 2551-2552 2553-2554 2555-2556 2557-2558 2559-2560 2561-2562 2563-2564 2565-2566 2567-2568 2569-2570 2571-2572 2573-2574 2575-2576 2577-2578 2579-2580 2581-2582 2583-2584 2585-2586 2587-2588 2589-2590 2591-2592 2593-2594 2595-2596 2597-2598 2599-2600 2601-2602 2603-2604 2605-2606 2607-2608 2609-2610 2611-2612 2613-2614 2615-2616 2617-2618 2619-2620 2621-2622 2623-2624 2625-2626 2627-2628 2629-2630 2631-2632 2633-2634 2635-2636 2637-2638 2639-2640 2641-2642 2643-2644 2645-2646 2647-2648 2649-2650 2651-2652 2653-2654 2655-2656 2657-2658 2659-2660 2661-2662 2663-2664 2665-2666 2667-2668 2669-2670 2671-2672 2673-2674 2675-2676 2677-2678 2679-2680 2681-2682 2683-2684 2685-2686 2687-2688 2689-2690 2691-2692 2693-2694 2695-2696 2697-2698 2699-2700 2701-2702 2703-2704 2705-2706 2707-2708 2709-2710 2711-2712 2713-2714 2715-2716 2717-2718 2719-2720 2721-2722 2723-2724 2725-2726 2727-2728 2729-2730 2731-2732 2733-2734 2735-2736 2737-2738 2739-2740 2741-2742 2743-2744 2745-2746 2747-2748 2749-2750 2751-2752 2753-2754 2755-2756 2757-2758 2759-2760 2761-2762 2763-2764 2765-2766 2767-2768 2769-2770 2771-2772 2773-2774 2775-2776 2777-2778 2779-2780 2781-2782 2783-2784 2785-2786 2787-2788 2789-2790 2791-2792 2793-2794 2795-2796 2797-2798 2799-2800 2801-2802 2803-2804 2805

33-24707-1000

[illegible]

1. The first step is to identify the problem or question that needs to be answered. This involves understanding the context and the specific requirements of the task.

...and the ... ..

Source: *Journal of the American Statistical Association*, 1964, 59, 1, 1-11.

• **Prevalence** = proportion of people with a disease at a particular point in time

100-443887-1000

[illegible]

Figure 1. The effect of the concentration of the *Agaricus bisporus* spores on the growth of *Agaricus bisporus* and *Agaricus bisporus* spores on the growth of *Agaricus bisporus* spores.

1. *Chlorophyll a* and *Chlorophyll b* were determined by the method of Arar and Collins (1971).  
 2. *Chlorophyll a* and *Chlorophyll b* were determined by the method of Arar and Collins (1971).

$$E_{\text{eff}} = \frac{\sum_{i=1}^n E_i}{n}$$

Journal of Interpersonal Violence 26(1) 2011 © The Author(s) 2011. Reprints and permissions: [sagepub.com/journalsPermissions.nav](http://sagepub.com/journalsPermissions.nav)

[illegible][illegible]

1. *Journal of the American Medical Association*, 1997; 277: 1033-1036.

4. Category - unclassified (U) (100-100000)

[illegible]

0907, de-af, R18066 de-af p. 2

... ..

[illegible]

the 1990s, the number of people in the world who are illiterate has increased from 400 million to 600 million. The number of illiterate people in the world is expected to increase to 700 million by the year 2015. The number of illiterate people in the world is expected to increase to 800 million by the year 2020. The number of illiterate people in the world is expected to increase to 900 million by the year 2025. The number of illiterate people in the world is expected to increase to 1 billion by the year 2030. The number of illiterate people in the world is expected to increase to 1.1 billion by the year 2035. The number of illiterate people in the world is expected to increase to 1.2 billion by the year 2040. The number of illiterate people in the world is expected to increase to 1.3 billion by the year 2045. The number of illiterate people in the world is expected to increase to 1.4 billion by the year 2050. The number of illiterate people in the world is expected to increase to 1.5 billion by the year 2055. The number of illiterate people in the world is expected to increase to 1.6 billion by the year 2060. The number of illiterate people in the world is expected to increase to 1.7 billion by the year 2065. The number of illiterate people in the world is expected to increase to 1.8 billion by the year 2070. The number of illiterate people in the world is expected to increase to 1.9 billion by the year 2075. The number of illiterate people in the world is expected to increase to 2 billion by the year 2080. The number of illiterate people in the world is expected to increase to 2.1 billion by the year 2085. The number of illiterate people in the world is expected to increase to 2.2 billion by the year 2090. The number of illiterate people in the world is expected to increase to 2.3 billion by the year 2095. The number of illiterate people in the world is expected to increase to 2.4 billion by the year 2100.

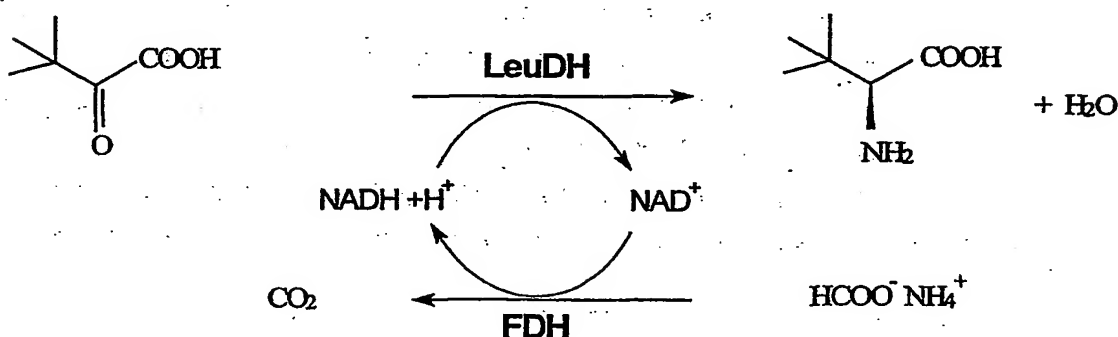
### Use of malate dehydrogenase for NADH regeneration

The present invention relates to a process for the preparation of enantiomerically enriched organic compounds. In particular, the present invention relates to an enzymatically operating process, in which, in a coupled enzymatic reaction system, NAD(P)H is consumed by one enzyme for the preparation of the organic compound and the NAD(P)H is simultaneously regenerated by a second enzyme system.

- 10 A reaction system which operates according to the invention in this manner and an advantageous whole cell catalyst or suitable plasmids are also proposed.

The production of optically active organic compounds, e.g. alcohols and amino acids, by a biocatalytic route is increasingly gaining importance. The coupled use of two dehydrogenases with cofactor regeneration has emerged, inter alia, as a route for the large-scale industrial synthesis of these compounds, in particular alcohols and amino acids (DE19753350, EP118750).

- 20 Equation 1:



- 25 *In situ* regeneration of NADH with the NAD-dependent formate dehydrogenase from *Candida boidinii* in the reductive amination of trimethyl pyruvate to give L-tert-leucine (Bommarius et al. Tetrahedron Asymmetry 1995, 6, 2851-2888).

In addition to their catalytic property and efficiency, the biocatalysts efficiently employed in an aqueous medium furthermore have the advantage that in contrast to a large number of synthetic metal-containing catalysts, the use of metal-containing starting substances, in particular those which contain heavy metals and are therefore toxic, can be dispensed with. The use of expensive and furthermore hazardous reducing agents, such as, for example, borane, in the case of asymmetric reduction can also be dispensed with.

The FDH, e.g. from *Candida boidinii*, successfully employed to date in these systems has the disadvantage that the specific activity of this enzyme class at 4-8 U/mg is very low. This necessitates the use of a large amount of expensive enzyme with recycling which is difficult from the apparatus point of view if a process designed in this way is to be carried out advantageously under economic aspects on an industrial scale.

Malate dehydrogenase (MDH) called "malic enzyme" catalyses the oxidative decarboxylation of malate to pyruvate. Numerous malate dehydrogenases from various organisms are known, thus, inter alia, from higher animals, plants and microorganisms. A distinction is made between four types of malate dehydrogenases, which are classified into the enzyme classes E.C. 1.1.1.37 to E.C. 1.1.1.40 (<http://www.genome.ad.jp>). NAD and/or NADP is required as a cofactor, depending on the type of malate dehydrogenase.

On the basis of the irreversibility of the oxidative decarboxylation reaction of L-malic acid to pyruvate, the use of malate dehydrogenase is also appropriate in the systems described above in respect of a favourable cofactor regeneration.

The use of malate dehydrogenase for regeneration of NAD is described, for example, by Suye et al. in a work from 1992 (S.-I. Suye, M. Kawagoe, S. Inuta, *Can. J. Chem. Eng.* 1992,

70, 306-312). NADH regeneration by means of malate dehydrogenase is used here for reductive amination of pyruvate by means of an alanine dehydrogenase, NADH being consumed. The pyruvate is formed by oxidative  
5 decarboxylation from L-malic acid, and is immediately consumed again in the following step by alanine dehydrogenase. A concentration of pyruvate in the reaction solution is therefore avoided, whereby the problem of any inhibitions of the enzymes involved by the presence of  
10 amounts of pyruvate in the stoichiometric range is also eliminated. Nevertheless, the system described is limited exclusively to the production of alanine (see also S.-I. Suje, *Recent Res. Devel. Ferment. Bioeng.* 1998, 1, 55-64).

Interestingly - in spite of the technically high potential  
15 - apart from these works by the Suje study group no further works using "malate dehydrogenase" for regeneration of the oxidized cofactor NAD<sup>+</sup> during reductions of ketones or reductive aminations of keto acids have been described.

The object of the present invention was therefore to  
20 provide a further process for the preparation of chiral organic compounds, such as amino acids or alcohols, which can be obtained by a coupled enzymatic reaction system as described above, using malate dehydrogenase, which is not limited to the preparation of one substance. In particular,  
25 it should be possible to employ this process on an industrial scale particularly advantageously under economic and ecological aspects.

This object is achieved by a process with the characterizing features of claim 1 of the present  
30 invention. Claims 2 to 7 relate to preferred embodiments. Claims 8 and 9 protect a reaction system according to the invention and a correspondingly operating whole cell catalyst. Claim 10 protects preferred plasmids.

In a process for the preparation of enantiomerically enriched organic compounds in a coupled enzymatic reaction system comprising a first enzymatic transformation of an organic substrate, NAD(P)H being consumed, and the  
5 regeneration of the NAD(P)H in a second enzymatic transformation by a malate dehydrogenase, with oxidation of L-malic acid to pyruvate and CO<sub>2</sub>, as a result of not employing the pyruvate formed from the second enzymatic transformation as the substrate in the first enzymatic  
10 transformation, the object described is achieved in an entirely surprising but in return no less advantageous manner. It can be regarded entirely as surprising that the simultaneous use e.g. of an amino acid dehydrogenase with malate dehydrogenase, which has a decarboxylating action,  
15 is possible without problems without the occurrence of cross-reactions. In addition, no inhibiting influences of substrates and products of the primary reaction on the malate dehydrogenase and vice versa can be detected. In particular, it is a positive feature that the pyruvate  
20 formed as the by-product does not have an inhibiting action on the malate dehydrogenase itself or on the alcohol or amino acid dehydrogenases employed in parallel.

Enantiomerically enriched alcohols or amino acids are advantageously prepared with the process according to the  
25 invention. In this case, inexpensive alcohol dehydrogenase or amino acid dehydrogenase which are known universally are possible as enzymes for the first enzymatic transformation. The expert is in principle free in the choice thereof, which is made according to the nature of the substrate  
30 spectrum, stability and rate of conversion of the enzyme in question. Known enzymes of this origin are described in K. Drauz, H. Waldmann (eds.), Enzyme Catalysis in Organic Synthesis, volume III, Wiley-VCH, Weinheim, 2002, chapter 15.

35 The use of the alcohol dehydrogenase from the organisms *Rhodococcus erythropolis* (S-ADH) or *Lactobacillus kefir* (R-

ADH) (ADH from *R. erythropolis*: J. Peters, T. Zelinski, M.-R. Kula, Purification and characterization of a novel carbonyl reductase silated from *Rhodococcus erythropolis*, J. Biotechnol. 1994, 33, 283-292) (ADH from *Lactobacillus* 5 *kefir*: C. W. Bradshaw, W. Hummel, C.-H. Wong, *Lactobacillus kefir* Alcohol Dehydrogenase: A Useful Catalyst for Synthesis, J. Org. Chem. 1992, 57, 1532-1536.) is particularly advantageous. In respect of preferred amino acid dehydrogenases, the expert is referred to, for 10 example, leucine dehydrogenases or phenylalanine dehydrogenases (A. Bommarius in: Enzyme Catalysis in Organic Synthesis (eds.: K. Drauz, H. Waldmann), volume III, Wiley-VCH, Weinheim, 2002, chapter 15.3).

Malate dehydrogenases are also familiar to the expert (lit. 15 see above or the dissertation by S. Naamnieh, University of Düsseldorf, in preparation). Here also, the expert will choose the dehydrogenase which can be employed most efficiently for his purpose. In principle, those malate dehydrogenases which regenerate the NAD(P)H in an extent 20 such that no bottleneck arises for the course of the reaction of the other enzyme employed.

The known malate dehydrogenase from *E. coli* K12 is preferred in this connection. Gene isolation and cloning are described in S. Naamnieh's dissertation, University of 25 Düsseldorf, in preparation p. 70 et seq.

In principle, the process according to the invention can be carried out in purely aqueous solution. However, it is also possible to add any desired parts of a water-soluble organic solvent to the aqueous solution in order e.g. to 30 optimize the reaction in respect of poorly water-soluble substrates. Possible such solvents are, in particular, ethylene glycol, DME or glycerol.

However, multi-phase, in particular two-phase systems comprising an aqueous phase can furthermore also serve as 35 the solvent mixture for the process according to the

invention. The use of certain solvents which are not water-soluble has already proved itself here (DE10233107). The statements made there in this respect also apply here accordingly.

5 The expert in principle has a free choice of the temperature present during the reaction. He preferably directs himself towards obtaining a highest possible yield of product in the highest possible purity in the shortest possible time. Furthermore, the enzymes employed should be  
10 sufficiently stable under the temperatures employed and the reaction should proceed with the highest possible enantioselectivity. In respect of the use of enzymes from thermophilic organisms, it is entirely possible for temperatures of 100°C to represent the upper limit of the  
15 temperature range in the reaction. -15°C is certainly appropriate as the lower limit in aqueous systems. A temperature interval between 10 and 60, particularly preferably between 20 and 40°C is advantageously to be established.

20 The pH during the reaction is determined by the expert from the enzyme stabilities and conversion rates and is adjusted accordingly for the process according to the invention. For the malate dehydrogenase from *E. coli* it has been found that the optimum pH is > 10. In general, the range

25 preferred for enzymes from pH 5 to 11 is chosen. A pH range from 5.5 to 10.0, in particular 6.0 to 9.0, can preferably be present.

The invention also provides a coupled enzymatic reaction system for the preparation of enantiomerically enriched  
30 organic compounds, comprising a first enzymatic transformation of an organic substrate, NAD(P)H being consumed, and the regeneration of the NAD(P)H in a second enzymatic transformation by a malate dehydrogenase, with oxidation of L-malic acid to pyruvate and CO<sub>2</sub>, wherein the  
35 pyruvate formed from the second enzymatic transformation is



not employed as the substrate in the first enzymatic transformation. The same advantages and preferred embodiments as have already been mentioned in respect of the process according to the invention apply in principle to this reaction system.

- The reaction system is advantageously employed, for example, in a stirred tank, a cascade of stirred tanks or in membrane reactors, which can be operated both in batch operation and continuously.
- 10 In the context of the invention, membrane reactor is understood as meaning any reaction vessel in which the catalyst is enclosed in a reactor, while low molecular weight substances are fed to the reactor or can leave it. The membrane here can be integrated directly into the
- 15 reaction space or incorporated outside in a separate filtration module, in which the reaction solution flows continuously or intermittently through the filtration module and the retained product is recycled into the reactor. Suitable embodiments are described, inter alia, in
- 20 WO98/22415 and in Wandrey et al. in Yearbook 1998, Verfahrenstechnik und Chemieingenieurwesen, VDI p. 151 et seq.; Wandrey et al. in Applied Homogeneous Catalysis with Organometallic Compounds, vol. 2, VCH 1996, p. 832 et seq.; Kragl et al., Angew. Chem. 1996, 6, 684 et seq.
- 25 The continuous procedure which is possible in this apparatus, in addition to the batch and semi-continuous procedure, can be carried out here as desired in the cross-flow filtration mode (fig. 3) or as dead-end filtration (fig. 2). Both process variants are described in principle
- 30 in the prior art (Engineering Processes for Bioseparations, ed.: L.R. Weatherley, Heinemann, 1994, 135-165; Wandrey et al., Tetrahedron Asymmetry 1999, 10, 923-928).

The present invention also provides whole cell catalysts comprising a cloned gene for a first enzyme for

35 transformation of an organic substrate and a cloned gene for a malate dehydrogenase, these being capable of

preparation of an enantiomerically enriched organic compound in a first enzymatic transformation, NAD(P)H being consumed, and of allowing the regeneration of the NAD(P)H to take place in a second enzymatic transformation by malate dehydrogenase, with oxidation of L-malic acid to pyruvate and CO<sub>2</sub>, wherein the pyruvate formed from the second enzymatic transformation is not employed as the substrate in the first enzymatic transformation.

The whole cell catalyst according to the invention preferably has an enzyme (polypeptide) with amino acid or alcohol dehydrogenase activity and one with malate dehydrogenase activity, originating in particular from the organisms mentioned above.

Microorganisms which can be used are in principle all the organisms possible to the expert for this purpose, such as e.g. yeasts, such as *Hansenula polymorpha*, *Pichia* sp. and *Saccharomyces cerevisiae*, prokaryotes, such as *E. coli* and *Bacillus subtilis* or eukaryotes, such as mammalian cells and insect cells. *E. coli* strains are preferably to be used for this purpose. The following are very particularly preferred: *E. coli* XL1-Blue, NM522, JM101, JM109, JM105, RR1, DH5 $\alpha$ , TOP10 or HB101.

An organism as mentioned in DE10155928 is preferably employed as the host organism.

The advantage of such an organism is the simultaneous expression of both polypeptide systems, which means that only one recombinant organism has to be cultured for the reaction according to the invention.

To coordinate the expression of the polypeptides in respect of their rates of conversion, the corresponding coding nucleic acid sequences can be accommodated on different plasmids with different numbers of copies and/or promoters of different potency can be used for an expression of the nucleic acid sequences of different intensity. In such coordinated enzyme systems, advantageously no accumulation of an intermediate compound occurs, and the reaction in question can proceed at an optimum overall rate. However,

this is adequately known to the expert (Gellissen, G.; Piontek, M.; Dahlems, U.; Jenzelewski, V.; Gavagan, J. W.; DiCosimo, R.; Anton, D. L.; Janowicz, Z. A. (1996), Recombinant *Hansenula polymorpha* as a biocatalyst.

- 5 Coexpression of the spinach glycolate oxidase (GO) and the *S. cerevisiae* catalase T (CTT1) gene, Appl. Microbiol. Biotechnol. 46, 46-54; Farwick, M.; London, M.; Dohmen, J.; Dahlems, U.; Gellissen, G.; Strasser, A. W.; DE19920712). It is very particularly advantageous that the whole cell  
10 catalyst according to the invention optionally further metabolizes the pyruvate formed in the reaction according to the invention, as it may use it as a nutrient source. Whole cell catalysts designed in this manner mean that the pyruvate is not obtained as a by-product of the reaction  
15 and therefore also does not have to be separated off from the chiral product actually desired in further process steps.

- The preparation of the whole cell catalyst can in principle be carried out by measures known to the expert (Sambrook,  
20 J.; Fritsch, E. F. and Maniatis, T. (1989), Molecular cloning: a laboratory manual, 2nd ed., Cold Spring Harbor Laboratory Press, New York; Balbas, P. and Bolivar, F. (1990), Design and construction of expression plasmid vectors in *E. coli*, Methods Enzymol. 185, 14-37; Rodriguez,  
25 R.L. and Denhardt, D. T (eds) (1988), Vectors: a survey of molecular cloning vectors and their uses, 205-225, Butterworth, Stoneham). In respect of the general procedure (PCR, cloning, expression etc.) reference may also be made to the following literature and that cited there: Universal  
30 GenomeWalker™ Kit User Manual, Clontech, 3/2000 and literature cited there; Triglia T.; Peterson, M. G. and Kemp, D.J. (1988), A procedure for in vitro amplification of DNA segments that lie outside the boundaries of known sequences, Nucleic Acids Res. 16, 8186; Sambrook, J.;  
35 Fritsch, E. F. and Maniatis, T. (1989), Molecular cloning: a laboratory manual, 2nd ed., Cold Spring Harbor Laboratory Press, New York; Rodriguez, R.L. and Denhardt, D. T (eds)

(1988), Vectors: a survey of molecular cloning vectors and their uses, Butterworth, Stoneham.

The invention also provides plasmids containing gene constructs in which the gene for a malate dehydrogenase and  
5 a gene for an enzyme for transformation of an organic substrate with consumption of NAD(P)H are present.

Possible plasmids or vectors of origin are in principle all the embodiments available to the expert for this purpose.

Such plasmids and vectors can be found e.g. in Studier and

10 colleagues (Studier, W. F.; Rosenberg A. H.; Dunn J. J.; Dubendorff J. W.; (1990), Use of the T7 RNA polymerase to direct expression of cloned genes, Methods Enzymol. 185, 61-89) or the brochures of Novagen, Promega, New England Biolabs, Clontech or Gibco BRL. Further preferred plasmids

15 and vectors can be found in: Glover, D. M. (1985), DNA cloning: A Practical Approach, vol. I-III, IRL Press Ltd., Oxford; Rodriguez, R.L. and Denhardt, D. T (eds) (1988), Vectors: a survey of molecular cloning vectors and their uses, 179-204, Butterworth, Stoneham; Goeddel, D. V.

20 (1990), Systems for heterologous gene expression, Methods Enzymol. 185, 3-7; Sambrook, J.; Fritsch, E. F. and Maniatis, T. (1989), Molecular cloning: a laboratory manual, 2<sup>nd</sup> ed., Cold Spring Harbor Laboratory Press, New York.

25 Plasmids with which the gene construct containing the nucleic acids according to the invention can be cloned into the host organism in a very preferred manner are: pUC18 (Roche Biochemicals), pKK-177-3H (Roche Biochemicals), pBTac2 (Roche Biochemicals), pKK223-3 (Amersham Pharmacia  
30 Biotech), pKK-233-3 (Stratagene) or pET (Novagen). The plasmid pkk/phe/mali (fig. 5) is advantageous in this connection.

The preparation of this plasmid and of a corresponding recommicroorganism is described in the dissertation by S.

35 Naamnieh, University of Düsseldorf, in preparation p. 70 et seq.

For the use, the polypeptides in question of the process according to the invention can be used in the free form as homogeneously purified compounds or as an enzyme prepared by a recombinant method. These polypeptides can furthermore  
5 also be employed as a constituent of an intact guest organism or in combination with the broken-down cell mass of the host organism, which has been purified to any desired extent.

The use of the enzymes in immobilized form is also possible  
10 (Sharma B. P.; Bailey L. F. and Messing R. A. (1982), Immobilisierte Biomaterialien - Techniken und Anwendungen, Angew. Chem. 94, 836-852). The immobilization is advantageously carried out by lyophilization (Paradkar, V. M.; Dordick, J. S. (1994), Aqueous-Like Activity of  $\alpha$ -  
15 Chymotrypsin Dissolved in Nearly Anhydrous Organic Solvents, J. Am. Chem. Soc. 116, 5009-5010; Mori, T.; Okahata, Y. (1997), A variety of lipi-coated glycoside hydrolases as effective glycosyl transfer catalysts in homogeneous organic solvents, Tetrahedron Lett. 38, 1971-  
20 1974; Otamiri, M.; Adlercreutz, P.; Matthiasson, B. (1992), Complex formation between chymotrypsin and ethyl cellulose as a means to solubilize the enzyme in active form in toluene, Biocatalysis 6, 291-305). Lyophilization in the presence of surface-active substances, such as Aerosol OT  
25 or polyvinylpyrrolidone or polyethylene glycol (PEG) or Brij 52 (diethylene glycol monocetyl ether) (Kamiya, N.; Okazaki, S.-Y.; Goto, M. (1997), Surfactant-horseradish peroxidase complex catalytically active in anhydrous benzene, Biotechnol. Tech. 11, 375-378), is very  
30 particularly preferred.

Immobilization on Eupergit<sup>®</sup>, in particular Eupergit C<sup>®</sup> and Eupergit 250L<sup>®</sup> (Röhm) (Eupergit.RTM. C, a carrier for immobilization of enzymes of industrial potential. Katchalski-Katzir, E.; Kraemer, D. M. Journal of Molecular  
35 Catalysis B: Enzymatic (2000), 10(1-3), 157-176), is extremely preferred.

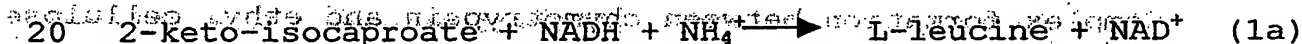
Immobilization on Ni-NTA in combination with the

polypeptide supplemented with the His tag (hexa-histidine) is likewise preferred (Purification of proteins using polyhistidine affinity tags. Bornhorst, Joshua A.; Falke, Joseph J. Methods in Enzymology (2000), 326, 245-254).

5 The use as CLECs is also conceivable (St. Clair, N.; Wang, Y.-F.; Margolin, A. L. (2000), Cofactor-bound cross-linked enzyme crystals (CLEC) of alcohol dehydrogenase, Angew. Chem. Int. Ed. 39, 380-383).

By these measures it can be possible to generate from  
10 polypeptides which become unstable due to organic solvents those which can operate in mixtures of aqueous and organic solvents or entirely in organic media.

The process according to the invention can be carried out such that the MDH from *E. coli* is coupled with an NAD-  
15 dependent leucine dehydrogenase (LeuDH from *Bacillus cereus*; Sigma). LeuDH catalyses the reductive amination of aliphatic keto acids, such as, for example, ketoisocaproate, to the corresponding L-amino acids, such as L-leucine, with consumption of NADH (equation (1)).



The course of the reaction was monitored by HPLC. The result can be seen from the following table 1 in comparison with an equivalent conversion with FDH instead of MDH.

Table 1: Comparison of the formation of L-leucine (HPLC) in a coupled batch with coenzyme regeneration by MDH and FDH. In each case 10 mM ketoisocaproate and for the regeneration by malate dehydrogenase (MDH) 100 mM L-malate or by formate dehydrogenase (FDH) 100 mM formate were employed.

Time [ min]	L-Leucine with MDH [ mM]	L-Leucine with FDH [ mM]
0	0.7	0.1
10	8.8	9.9
30	9.6	11.1
60	10.8	9.8
120	9.9	9.9

The use of alcohol dehydrogenases in combination with malate dehydrogenases was also investigated. The MDH from *E. coli* is coupled with an NAD-dependent S-specific alcohol dehydrogenase from *Rhodococcus erythropolis* (RE-ADH; DE10218689). The usability of the MDH is tested here via the reduction of a ketone (p-Cl-acetophenone = pCAp) in accordance with equation (4).

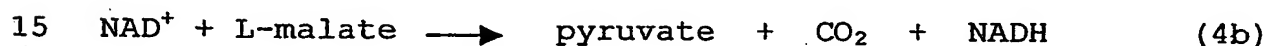
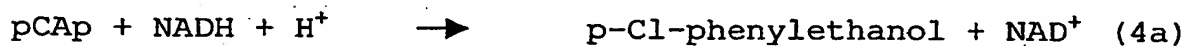


Table 2: Decrease in the ketone p-Cl-acetophenone (employed 10 mM) and increase in the enzymatically formed alcohol p-Cl-phenylethanol (100%, corresp. to 10 mM) as a function of time.

Time [ min ]	Ketone [ % ]	Alcohol [ % ]
0	100	0
10	28	72
20	12	88
30	4	96
60	0	100

5

Enantiomerically enriched or enantiomer-enriched describes the fact that one optical antipode is present in a mixture with its other to >50%.

If one stereo-centre is present the structures shown relate to the two possible enantiomers, and if more than one stereo-centre is present in the molecule they relate to all the possible diastereomers and, in respect of a diastereomer, to the two possible enantiomers of the compound in question which fall under this.

15 The organism *Candida boidinii* is deposited under number ATCC 32195 at the American Type Culture Collection and is accessible to the public.

The documents of the prior art mentioned in this specification are regarded as also included in the disclosure.

20



Descriptions of the drawings:

Fig. 2 shows a membrane reactor with dead-end filtration. The substrate 1 is transferred via a pump 2 into the reactor space 3, which contains a membrane 5. In the reactor space, which is operated with a stirrer, are, in addition to the solvent, the catalyst 4, the product 6 and unreacted substrate 1. Low molecular weight 6 is chiefly filtered off via the membrane 5.

Fig. 3 shows a membrane reactor with cross-flow filtration. The substrate 7 is transferred here via the pump 8 into the stirred reactor space, in which is also solvent, catalyst 9 and product 14. A solvent flow which leads via a heat exchanger 12, which may be present, into the cross-flow filtration cell 15 is established via the pump 16. The low molecular weight product 14 is separated off here via the membrane 13. High molecular weight catalyst 9 is then passed back with the solvent flow, if appropriate via a heat exchanger 12 again, if appropriate via the valve 11, into the reactor 10.

## Example 1:

For production of the MDH from *E. coli* K12 used here, see the dissertation by S. Naamnieh, University of Düsseldorf, in preparation, p. 70 et seq.

5. Purification and biochemical properties of the MDH from *E. coli*:

## a) Purification

The purification of the recMDH from *E. coli* crude extracts (expression strain: *E. coli* derivative JM105) was carried out in accordance with purification protocol (Stols L., and Donnelly M. I. (1997). Production of succinic acid through overexpression of NAD(+)-dependent malic enzyme in an *Escherichia coli* mutant. *Appl Environ Microbiol* 63: 2695-701.). The rec-bacteria cells were first broken down by disintegration with glass beads (breakdown buffer Tris/HCl 100 mM pH 7.5). Thereafter, a purification step by a Q-Sepharose was carried out. After the purification by means of Q-Sepharose, it was possible to determine a specific activity of the MDH of about 7.3 U/mg. By purification of this enzyme by means of further chromatography steps, hydroxyapatite and Phenylsepharose, it was possible to purify the MDH to homogeneity with a specific activity of 133 U/mg.

Tab. 3: Summary of the purification of the rec-MDH from *E. coli*.

Purification step	Activity (U)	Protein (mg)	Spec. act. (U/mg)	Enrichment (-fold)	Yield (%)
Crude extract	210	202	1.03		100
Q-Sepharose	184	25.2	7.3	7.1	88
Hydroxy-apatite	68	1.6	42.5	41	32
Phenyl-sepharose	24	0.18	133	129	11

b) Biochemical characterization

5 - Km values

For L-malate a Km value of 0.29 mM was measured, and for the coenzyme NAD<sup>+</sup> a Km value of 0.14 mM.

Both Km values lie in a low range of < 1 mM, and they show that the two substrates are recognized by the enzyme with a good affinity. The two values suggest that the MDH can be used for the regeneration of NADH.

- Optimum pH (fig. 1)

MDH shows a maximum activity at relatively high pH values of 11 and higher. Nevertheless, the drop in activity at lower pH values is relatively small, thus 72% activity is still present at pH 8.0, and 67% activity still at pH 7.0.

- Optimum temperature

The optimum temperature of the MDH is approx. 55°C (fig. 4).

Example 2:

- 5 a) Coupling of leucine dehydrogenase with malate dehydrogenase

The MDH from *E. coli* is coupled with an NAD-dependent leucine dehydrogenase (LeuDH from *Bacillus cereus*; Sigma). LeuDH catalyses the reductive amination of aliphatic keto  
10 acids, such as, for example, ketoisocaproate, to the corresponding L-amino acids, such as L-leucine, with consumption of NADH.

Test batch (1 ml in total; unless stated otherwise the concentration of the stock solution is stated in  
15 parentheses):

526 µl Hepes buffer (200 mM Hepes, pH 8.5 with 10 mM MgCl<sub>2</sub>); 143 µl ammonium sulfate solution (500 mM in the test); 100 µl ketoisocaproate (100 mM); 20 µl NAD<sup>+</sup> (50 mM);  
200 µl L-malate (500 mM, Na salt, dissolved in Hepes  
20 buffer, pH 8.5); 1 µl LeuDH (0.5 U in the test); 10 µl MDH (1.5 U in the test; partly purified).

The test batch is incubated at 30°C, and after 0, 10, 30, 60 and 120 min samples are taken (50 µl; Eppendorf reaction vessels) and heated for 3 min at 95°C to stop the reaction.

25 Denatured protein is separated off by centrifugation for 10 min at 13,000 rpm (Eppendorf bench centrifuge) and the supernatant is analysed by means of HPLC, after derivatization with ortho-phthalaldehyde (OPA).

Derivatization with OPA (= ortho-phthaldialdehyde):

30 140 µl Na borate buffer (100 mM; pH 10.4); 40 µl sample or standard; 20 µl OPA/IBLC reagent (= ortho-phthaldialdehyde

/ N-isobutyryl-L-cysteine). 20 µl of this reaction solution are injected for the HPLC analysis.

HPLC analysis:

For the results see table 1:

5

b) Comparison experiment: Coupling of leucine dehydrogenase with formate dehydrogenase

In a comparison batch carried out in parallel, the same components as described above were used, but instead of the  
10 malate dehydrogenase 0.5 U formate dehydrogenase (FDH from *Candida boidinii*; Sigma) was employed and instead of 100 mM L-malate 100 mM formate was used as the regeneration substrate.

For the results see table 1.

15

Example 3: Coupling of the MDH with alcohol dehydrogenase:

The MDH from *E. coli* (expression strain: *E. coli* derivative JM 105) is coupled with an NAD-dependent S-specific alcohol dehydrogenase from *Rhodococcus erythropolis* (RE-ADH). The  
20 usability of the MDH is tested here via the reduction of a ketone (p-Cl-acetophenone).

Test batch (1 ml in total; unless stated otherwise the concentration of the stock solution is stated in parentheses):

25 678.7 µl Hepes buffer (100 mM Hepes, pH 8.5 with 10 mM MgCl<sub>2</sub>); 1.3 µl p-Cl-acetophenone (10 mM in the test); 20 µl NAD<sup>+</sup> (50 mM); 200 µl L-malate (500 mM, Na salt, dissolved in Hepes buffer, pH 8.5); 15 µl RE-ADH (1 U in the test); 85 µl MDH (1 U in the test; partly purified).

The test batch is incubated at 30°C, and after 0, 10, 20, 30 and 60 min samples (50 µl) are taken, 100 µl ethyl acetate are added and the upper phase is analysed by means of gas chromatography for the formation of the alcohol p-Cl-phenylethanol.

For the results see table 2.

#### Example 4: Construction of an expression vector with heterologous expression

10 From the sequence of the amplified fragment, primers with integrated restriction cleavage sites and a codon for the ribosomal binding site were constructed. After amplification of the malate dehydrogenase from the recombinant pUC18, the PCR fragment was cloned into the  
15 recombinant recPhe-pKK-223-3 expression vector after the PheDH sequence at the PstI and HindIII restriction cleavage sites (fig. 8 - Construction of the plasmid for a heterologous expression for L-Phe synthesis by means of whole cell conversion).

#### 20 PCR:

5' forward: N<sup>6</sup>-malic-pst

5' CTGCAGAGCCCGAGGGATGGATATTCAAAAA 3'

concentration 100 pmol/µl

5' reverse: C<sup>6</sup>-malic-Hin

25 5' AAGCTTTTAGATGGAGGTACGGCGGTAGTC 3'

concentration 100 pmol/µl

Table 4: PCR protocol for amplification of the malate dehydrogenase from the recombinant pUC18 plasmid. The concentrations of the template DNA were varied.

Template DANN recpUC18	N' -malic- pst prim 1	C' -malic- Hin prim 2	dNTP	Buffer	Taq polymerase	H <sub>2</sub> O
50 ng/μl	1 μl	1 μl	2 μl	10 μl	1 μl	83 μl
25 ng/μl	1 μl	1 μl	2 μl	10 μl	1 μl	83 μl
10 ng/μl	1 μl	1 μl	2 μl	10 μl	1 μl	83 μl

5

One cycle consists of:

Denaturing step : 94°C

Annealing step: 59°C

Amplification step: 72°C

#### 10 Cloning:

The new construct of the recombinant plasmid (fig. 5) was transformed into competent *E. coli* cells JM 105 or HB 101.

The standard transformation was carried out in accordance with the protocol of Hanahan (Hanahan D. (1983). Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol* 166: 557-80.). For this, 100-200 μl of competent *E. coli* cells were thawed on ice and 40 ng DNA from the ligation batch were added. The plasmid cell suspension was cooled for 30 min on ice and then heated at 42°C for 90 sec and immediately cooled again on ice. After addition of 300 μl LB medium the cells were incubated for about 45 min at 37°C for regeneration. 200 μl of this culture were then

15

20

plated out on to an LB plate containing antibiotic and incubated overnight at 37°C.

With the cloning of the malate dehydrogenase 3' to the PheDH on the PstI and HindIII cleavage site with its own ribosomal binding site, expression of the two enzymes could take place simultaneously.

Note: Further detailed information on the experimental preparation of these cells will be described in: S.

Naamnieh, Dissertation, University of Düsseldorf, in preparation.

#### Example 5: Coexpression of PheDH and malate dehydrogenase

Of the positive clones, some were selected for expression. An individual colony of the particular clones was transinoculated into 5 ml LB<sub>amp</sub> medium, and after OD<sub>580</sub> = 0.6 was reached was induced with 1 mM IPTG. The induction was carried out overnight and the harvested cells were broken down with ultrasound.

The recombinant strain HB101 shows a malate dehydrogenase activity of 100 U/ml and likewise a PheDH activity of 130 U/ml. The activity of the two enzymes in the recombinant strain JM-105 is clearly higher and is at ~ 600 U/ml for malate dehydrogenase and ~ 1,200 U/ml for the PheDH.

The two recombinant strains were cultured in a 10 l fermenter and the activity of the two enzymes was determined.



Table 5: Determination of the activity of the expressed enzymes in a 10 l fermenter with LB medium as batch fermentation.

	Activity of PheDH		Activity of malate dehydrogenase	
	(U/ml)	(U/mg)	(U/ml)	(U/mg)
E. coli HB101	400	33	220	22
E. coli JM105	1300	118	640	71

- 5 It can be seen from the expression data that the *E. coli* strain JM 105 shows a significantly better activity for both enzymes, and all further experiments were therefore carried out with this strain.

10 Example 6: Optimization of the activity

To be able to utilize the maximum activity of the crude extracts obtained, several parameters were investigated and varied.

- 15 The two heterologously expressed enzymes show the best stability properties under different conditions. It was of interest to determine the optimum properties for both enzymes in the same system. The following experiments were carried out with a view to this fact.

a) Optimization of the breakdown buffer

- 20 1 g of cells JM<sub>105</sub> was broken down in 0.1 M Tris or 0.1 M Kpi buffer with/without BSA (1.5 g/l) 30%. The breakdown was carried out with ultrasound over 70 cont. cycles. In

addition to the buffers, 1.5 g/l BSA were added for stabilization of the enzymes.

Table 6: Comparison of the activity as a function of the breakdown buffer

	0.1 M Tris		0.1 M Kpi	
	- BSA	+ BSA	- BSA	+ BSA
PheDH	520 U/ml	610 U/ml	730 U/ml	1100 U/ml
Malate dehydrogenase	430 U/ml	720 U/ml	320 U/ml	610 U/ml

5

The addition of BSA led to an increase in the activity in both cases. The breakdown buffer also influenced the activities. It was to be seen here that the suitable breakdown buffer was different for the individual enzymes.

- 10 The Kpi buffer was more suitable for the PheDH than for the malate dehydrogenase, but since the decrease in activity of the malate dehydrogenase in the Kpi buffer was relatively low, the recombinant cells continued to be broken down in this buffer after the heterologous expression.

15

b) Duration of breakdown to investigate the stability

- The duration of the breakdown was also investigated and the critical point for stability of the enzymes during this operation was determined. The optimum duration of breakdown can be obtained from these data (fig. 9 - Stability determination of the PheDH and the malate dehydrogenase after various breakdown times by means of ultrasound. The cells were cooled intermediately for 30 seconds after a 60 and 30 second treatment.
- 20

The following suspension was used for this experiment:

1 g recombinant cells (JM105)

3 ml Kpi buffer 0.1 M

During longer treatment of the cells with ultrasound the activity of the PheDH decreases drastically, whereas the activity of the malate dehydrogenase is retained. The ideal breakdown conditions for a 25% breakdown of 1 g of recombinant *E. coli* JM105 are therefore 4 x 30 s ultrasound treatment with 3 x 30 s intermediate cooling in an ice-bath.

During longer treatments with ultrasound the sample is heated, which can lead to denaturing of the enzymes. The amount of protein was determined in this experiment and can be seen from the following.

Table 7: Protein determination of the two enzymes expressed, PheDH and malate dehydrogenase, after variation of the breakdown time. The cells were cooled intermediately for 30 seconds after a 60 and 30 second treatment.

	60 sec x 2 spec. activity: U/mg	30 sec x 4 spec. activity: U/mg	30 sec x 8 spec. activity: U/mg
PheDH	30	105	16
Malate dehydrogenase	20	90	110

With a purification of the malate dehydrogenase to homogeneity, it was possible to achieve a specific activity of 466 U/mg. The purification steps are summarized in table 8.

Table 8: Purification of the recombinant malate dehydrogenase

	Volume (ml)	Activity (U)	Protein (mg)	Spec. activity (U/mg)	Yield (%)	Factor
Ultracentrifugation	0.6	820	13	63	100	1
Hydroxyapatite	9	470	5	94	57	1.49
Q-Sepharose	7	496	2.1	236	60	3.74
Phenyl-sepharose	1.9	280	0.6	466	34	7.39

c)  $K_m$  value determination

5 The  $K_m$  values were determined for the substrate and the coenzyme of the malate dehydrogenase. The  $K_m$  values were determined on homogeneous or partly purified rec-malate dehydrogenase samples.

L-Malate: 0.29 mM

10 NAD<sup>+</sup> 0.14 mM

## Example 7: Coupled L-phenylalanine synthesis with regeneration of the coenzyme NADH

15 Important factors for a coupled reaction are the optimum pH and the heat stability of the two enzymes. In addition to the stability of the enzymes, further factors play a role, such as e.g. the influence of the various substrates on the enzymes.

In respect of the optimum pH, a kinetics study was conducted and the pH-dependency of the synthesis was determined. The increase in the activity at an increasing pH can be seen from fig. 10, a pH of 8.0, in which although the two enzymes do not show the highest activity, the coenzyme remains stable, being chosen for the synthesis for coenzyme stability reasons (fig. 10 - Optimum pH of malate dehydrogenase and PheDH. The activity of the two enzymes increases as the pH increases. The measurements were carried out with partly purified enzyme. For the phenylalanine dehydrogenase, the reductive amination was measured).

A second factor for the coupled enzyme reaction is the suitable temperature at which the two enzymes remain stable for a relatively long period of time. A further experiment was therefore carried out to determine the optimum temperature (fig. 11 - Optimum temperature. The measurements were carried out at pH 8.5 and in 0.1 M HEPES buffer).

As can be seen from fig. 11, the optimum temperature of both enzymes is 50°C. At 30°C the activity measured is only 60%.

The malate dehydrogenase is stable at 45°C for a relatively long period of time. However, since the PheDH becomes unstable at this temperature value, the syntheses were carried out at 30°C, so that it was possible to ensure the stability of both enzymes and of the coenzyme over a relatively long period of time.

Enzymes achieve their optimum activity in the particular suitable buffer. The two enzymes were tested with two different buffers in each case in a reaction batch (table 9).

Table 9: Comparison of the PheDH and malate dehydrogenase activity in various reaction buffers. The activities are to be seen in per cent of the optimum.

	0.1 M HEPES buffer (pH 8.0)	0.1 M Tris buffer (pH 8.0)
PheDH	84 %	100 %
MDH	100 %	42 %

5 Since the activity of the PheDH in HEPES buffer does not decrease considerably, the coupled enzyme reaction was carried out in this buffer.

With the determination of the buffer, pH and temperature values, it was possible to select suitable conditions and

10 media for the synthesis of phenylalanine by a coupled enzyme reaction with regeneration of the cofactor (NADH).

30 mM phenyl pyruvate, 100 mM ammonium sulfate, 100 mM HEPES buffer, 70 mM L-malate, 2 mM NAD<sup>+</sup>, 2 mM Mg<sup>2+</sup>, 25 U PheDH (partly purified) and 30 U malate dehydrogenase were

15 employed. The samples are analysed by means of HPLC.

The synthesis was monitored for several hours. After 4 h approx. 50% of the substrate employed, phenyl pyruvate, was converted into L-phenylalanine (fig. 12 - Formation kinetics for L-Phe. The formation of L-Phe was carried out

20 in situ).

#### Example 8: Whole cell conversion

The following medium was used as the standard batch for the whole cell conversion:

0.1 M	HEPES buffer (pH 8.0)
40 mM	phenyl pyruvate
0.1 M	L-malate
0.1 M	ammonium sulfate
5 2 mM	MgCl <sub>2</sub>

The conversion was carried out at 30°C and with 1 g of recombinant *E. coli* cells. The phenylalanine formed was detected by means of HPLC (fig. 6).

10 The formation of L-Phe by recombinant *E. coli* cells was monitored for 20 h and the yield was determined (fig. 7).

No metabolization of the product formed, L-Phe, was to be detected after incubation for 20 h.

## Patent claims:

1. Process for the preparation of enantiomerically enriched organic compounds in a coupled enzymatic reaction system, comprising a first enzymatic transformation of an organic substrate, NAD(P)H being consumed, and the regeneration of the NAD(P)H in a second enzymatic transformation by a malate dehydrogenase, with oxidation of L-malic acid to pyruvate and CO<sub>2</sub>,  
10 characterized in that the pyruvate formed from the second enzymatic transformation is not employed as the substrate in the first enzymatic transformation.
2. Process according to claim 1,  
15 characterized in that the first enzymatic transformation proceeds using an alcohol dehydrogenase or amino acid dehydrogenase.
3. Process according to claim 2,  
20 characterized in that an ADH from *Lactobacillus kefir* or *Rhodococcus erythropolis* is used as the alcohol dehydrogenase and a leucine dehydrogenase or phenylalanine dehydrogenase is used as the amino acid dehydrogenase.
4. Process according to one or more of the preceding  
25 claims characterized in that a malate dehydrogenase from *E. coli*, in particular *E. coli* K12, is used.
5. Process according to one or more of the preceding  
30 claims characterized in that the reaction is carried out in an aqueous single- or multi-phase solvent mixture.



6. Process according to one or more of the preceding claims,  
characterized in that  
the temperature during the reaction is between 20 and  
40°C.
7. Process according to one or more of the preceding claims,  
characterized in that  
the pH during the reaction is between 6 and 9.
8. Coupled enzymatic reaction system for the preparation  
of enantiomerically enriched organic compounds,  
comprising a first enzymatic transformation of an  
organic substrate, NAD(P)H being consumed, and the  
regeneration of the NAD(P)H in a second enzymatic  
transformation by a malate dehydrogenase, with  
oxidation of L-malic acid to pyruvate and CO<sub>2</sub>,  
characterized in that  
the pyruvate formed from the second enzymatic  
transformation is not employed as the substrate in the  
first enzymatic transformation.
9. Whole cell catalyst comprising a cloned gene for a  
first enzyme for transformation of an organic  
substrate and a cloned gene for a malate  
dehydrogenase, this being capable of preparation of an  
enantiomerically enriched organic compound in a first  
enzymatic transformation, NAD(P)H being consumed, and  
of allowing the regeneration of the NAD(P)H to take  
place in a second enzymatic transformation by malate  
dehydrogenase, with oxidation of L-malic acid to  
pyruvate and CO<sub>2</sub>, wherein the pyruvate formed from the  
second enzymatic transformation is not employed as the  
substrate in the first enzymatic transformation.
10. Plasmids containing gene constructs in which the gene  
for a malate dehydrogenase and a gene for an enzyme

for transformation of an organic substrate with consumption of NAD(P)H are present.

Fig. 1:

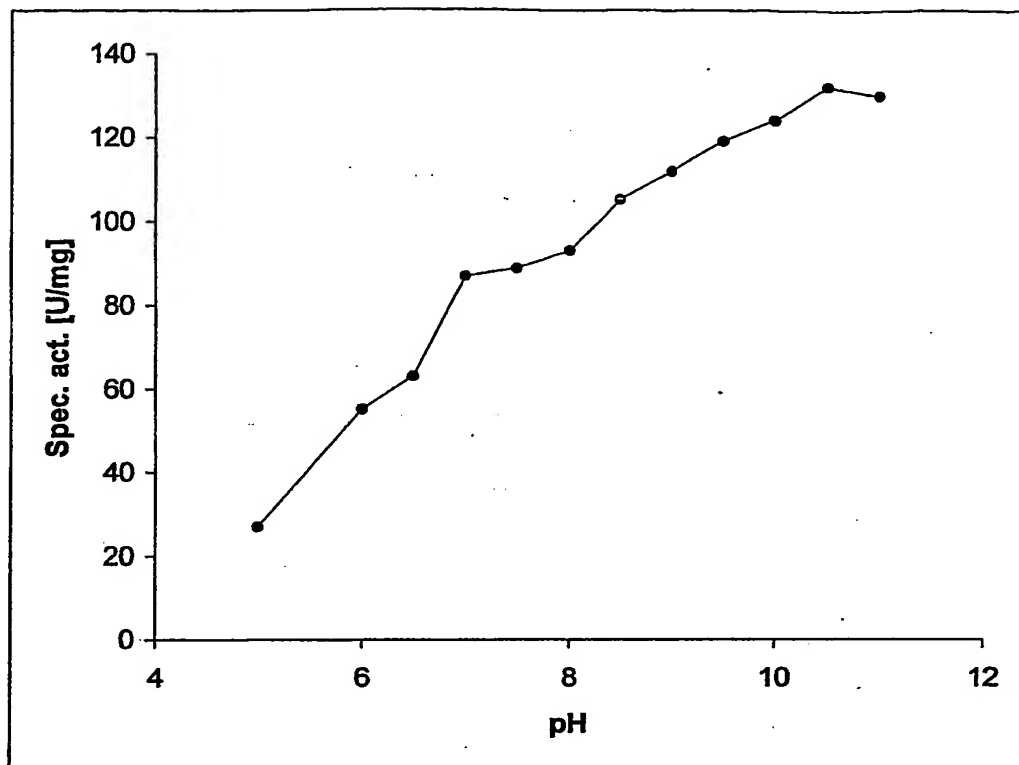




Fig. 2:

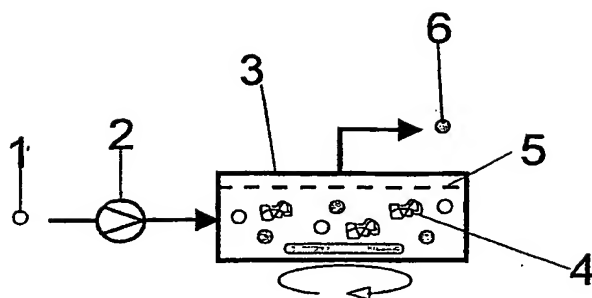
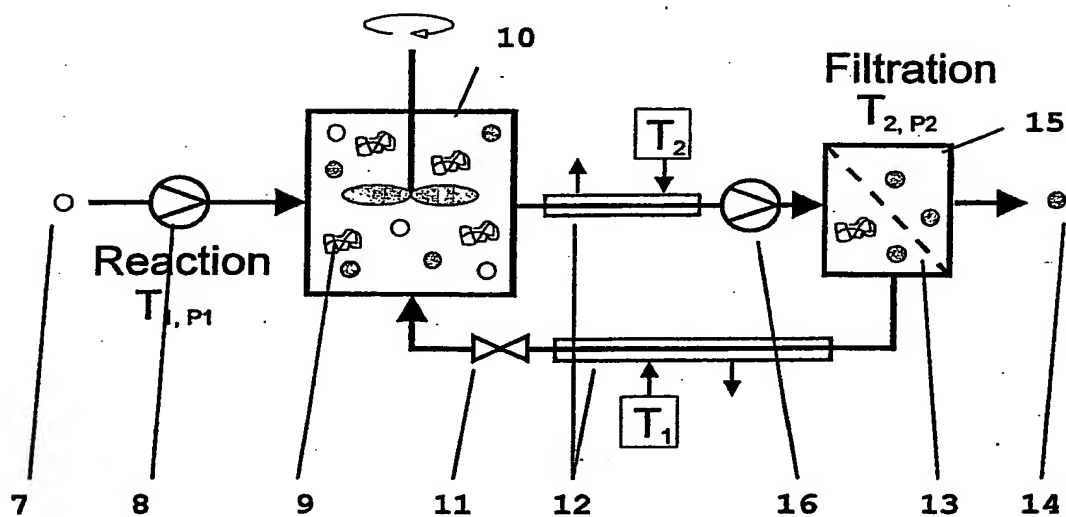


Fig. 3:



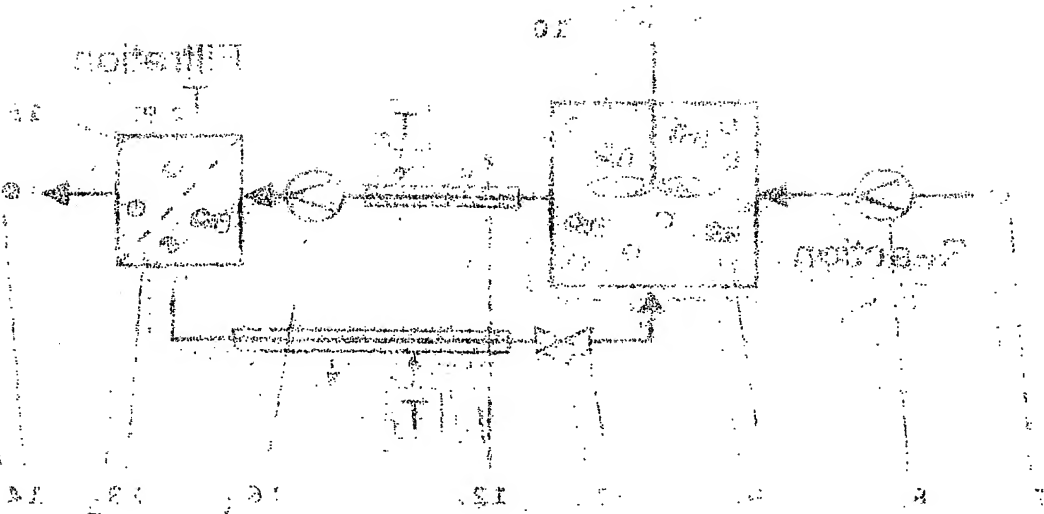
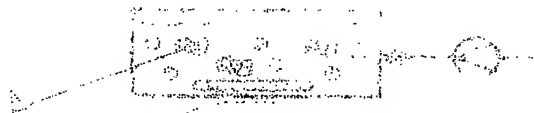
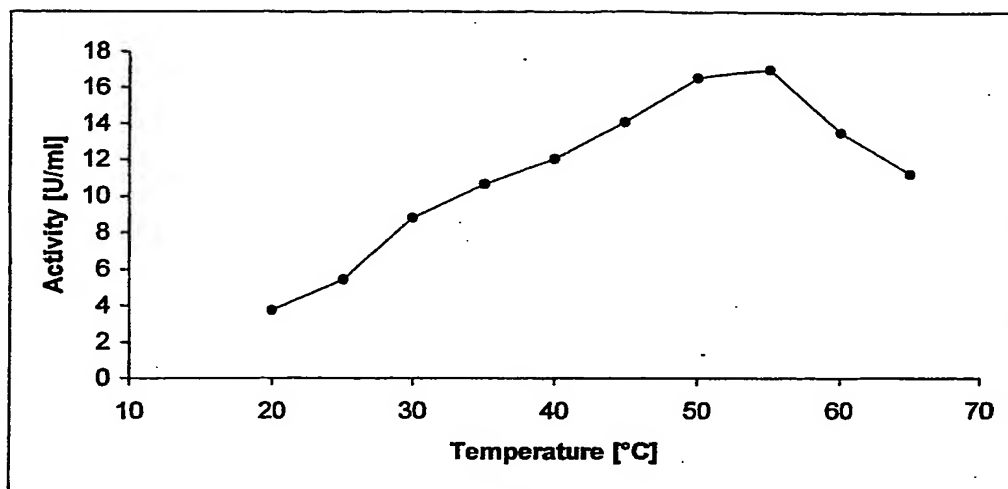


Fig. 4:



1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144	145	146	147	148	149	150	151	152	153	154	155	156	157	158	159	160	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180	181	182	183	184	185	186	187	188	189	190	191	192	193	194	195	196	197	198	199	200	201	202	203	204	205	206	207	208	209	210	211	212	213	214	215	216	217	218	219	220	221	222	223	224	225	226	227	228	229	230	231	232	233	234	235	236	237	238	239	240	241	242	243	244	245	246	247	248	249	250	251	252	253	254	255	256	257	258	259	260	261	262	263	264	265	266	267	268	269	270	271	272	273	274	275	276	277	278	279	280	281	282	283	284	285	286	287	288	289	290	291	292	293	294	295	296	297	298	299	300	301	302	303	304	305	306	307	308	309	310	311	312	313	314	315	316	317	318	319	320	321	322	323	324	325	326	327	328	329	330	331	332	333	334	335	336	337	338	339	340	341	342	343	344	345	346	347	348	349	350	351	352	353	354	355	356	357	358	359	360	361	362	363	364	365	366	367	368	369	370	371	372	373	374	375	376	377	378	379	380	381	382	383	384	385	386	387	388	389	390	391	392	393	394	395	396	397	398	399	400	401	402	403	404	405	406	407	408	409	410	411	412	413	414	415	416	417	418	419	420	421	422	423	424	425	426	427	428	429	430	431	432	433	434	435	436	437	438	439	440	441	442	443	444	445	446	447	448	449	450	451	452	453	454	455	456	457	458	459	460	461	462	463	464	465	466	467	468	469	470	471	472	473	474	475	476	477	478	479	480	481	482	483	484	485	486	487	488	489	490	491	492	493	494	495	496	497	498	499	500	501	502	503	504	505	506	507	508	509	510	511	512	513	514	515	516	517	518	519	520	521	522	523	524	525	526	527	528	529	530	531	532	533	534	535	536	537	538	539	540	541	542	543	544	545	546	547	548	549	550	551	552	553	554	555	556	557	558	559	560	561	562	563	564	565	566	567	568	569	570	571	572	573	574	575	576	577	578	579	580	581	582	583	584	585	586	587	588	589	590	591	592	593	594	595	596	597	598	599	600	601	602	603	604	605	606	607	608	609	610	611	612	613	614	615	616	617	618	619	620	621	622	623	624	625	626	627	628	629	630	631	632	633	634	635	636	637	638	639	640	641	642	643	644	645	646	647	648	649	650	651	652	653	654	655	656	657	658	659	660	661	662	663	664	665	666	667	668	669	670	671	672	673	674	675	676	677	678	679	680	681	682	683	684	685	686	687	688	689	690	691	692	693	694	695	696	697	698	699	700	701	702	703	704	705	706	707	708	709	710	711	712	713	714	715	716	717	718	719	720	721	722	723	724	725	726	727	728	729	730	731	732	733	734	735	736	737	738	739	740	741	742	743	744	745	746	747	748	749	750	751	752	753	754	755	756	757	758	759	760	761	762	763	764	765	766	767	768	769	770	771	772	773	774	775	776	777	778	779	780	781	782	783	784	785	786	787	788	789	790	791	792	793	794	795	796	797	798	799	800	801	802	803	804	805	806	807	808	809	810	811	812	813	814	815	816	817	818	819	820	821	822	823	824	825	826	827	828	829	830	831	832	833	834	835	836	837	838	839	840	841	842	843	844	845	846	847	848	849	850	851	852	853	854	855	856	857	858	859	860	861	862	863	864	865	866	867	868	869	870	871	872	873	874	875	876	877	878	879	880	881	882	883	884	885	886	887	888	889	890	891	892	893	894	895	896	897	898	899	900	901	902	903	904	905	906	907	908	909	910	911	912	913	914	915	916	917	918	919	920	921	922	923	924	925	926	927	928	929	930	931	932	933	934	935	936	937	938	939	940	941	942	943	944	945	946	947	948	949	950	951	952	953	954	955	956	957	958	959	960	961	962	963	964	965	966	967	968	969	970	971	972	973	974	975	976	977	978	979	980	981	982	983	984	985	986	987	988	989	990	991	992	993	994	995	996	997	998	999	1000	1001	1002	1003	1004	1005	1006	1007	1008	1009	1010	1011	1012	1013	1014	1015	1016	1017	1018	1019	1020	1021	1022	1023	1024	1025	1026	1027	1028	1029	1030	1031	1032	1033	1034	1035	1036	1037	1038	1039	1040	1041	1042	1043	1044	1045	1046	1047	1048	1049	1050	1051	1052	1053	1054	1055	1056	1057	1058	1059	1060	1061	1062	1063	1064	1065	1066	1067	1068	1069	1070	1071	1072	1073	1074	1075	1076	1077	1078	1079	1080	1081	1082	1083	1084	1085	1086	1087	1088	1089	1090	1091	1092	1093	1094	1095	1096	1097	1098	1099	1100	1101	1102	1103	1104	1105	1106	1107	1108	1109	1110	1111	1112	1113	1114	1115	1116	1117	1118	1119	1120	1121	1122	1123	1124	1125	1126	1127	1128	1129	1130	1131	1132	1133	1134	1135	1136	1137	1138	1139	1140	1141	1142	1143	1144	1145	1146	1147	1148	1149	1150	1151	1152	1153	1154	1155	1156	1157	1158	1159	1160	1161	1162	1163	1164	1165	1166	1167	1168	1169	1170	1171	1172	1173	1174	1175	1176	1177	1178	1179	1180	1181	1182	1183	1184	1185	1186	1187	1188	1189	1190	1191	1192	1193	1194	1195	1196	1197	1198	1199	1200	1201	1202	1203	1204	1205	1206	1207	1208	1209	1210	1211	1212	1213	1214	1215	1216	1217	1218	1219	1220	1221	1222	1223	1224	1225	1226	1227	1228	1229	1230	1231	1232	1233	1234	1235	1236	1237	1238	1239	1240	1241	1242	1243	1244	1245	1246	1247	1248	1249	1250	1251	1252	1253	1254	1255	1256	1257	1258	1259	1260	1261	1262	1263	1264	1265	1266	1267	1268	1269	1270	1271	1272	1273	1274	1275	1276	1277	1278	1279	1280	1281	1282	1283	1284	1285	1286	1287	1288	1289	1290	1291	1292	1293	1294	1295	1296	1297	1298	1299	1300	1301	1302	1303	1304	1305	1306	1307	1308	1309	1310	1311	1312	1313	1314	1315	1316	1317	1318	1319	1320	1321	1322	1323	1324	1325	1326	1327	1328	1329	1330	1331	1332	1333	1334	1335	1336	1337	1338	1339	1340	1341	1342	1343	1344	1345	1346	1347	1348	1349	1350	1351	1352	1353	1354	1355	1356	1357	1358	1359	1360	1361	1362	1363	1364	1365	1366	1367	1368	1369	1370	1371	1372	1373	1374	1375	1376	1377	1378	1379	1380	1381	1382	1383	1384	1385	1386	1387	1388	1389	1390	1391	1392	1393	1394	1395	1396	1397	1398	1399	1400	1401	1402	1403	1404	1405	1406	1407	1408	1409	1410	1411	1412	1413	1414	1415	1416	1417	1418	1419	1420	1421	1422	1423	1424	1425	1426	1427	1428	1429	1430	1431	1432	1433	1434	1435	1436	1437	1438	1439	1440	1441	1442	1443	1444	1445	1446	1447	1448	1449	1450	1451	1452	1453	1454	1455	1456	1457	1458	1459	1460	1461	1462	1463	1464	1465	1466	1467	1468	1469	1470	1471	1472	1473	1474	1475	1476	1477	1478	1479	1480	1481	1482	1483	1484	1485	1486	1487
---	---	---	---	---	---	---	---	---	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------



Fig. 5:

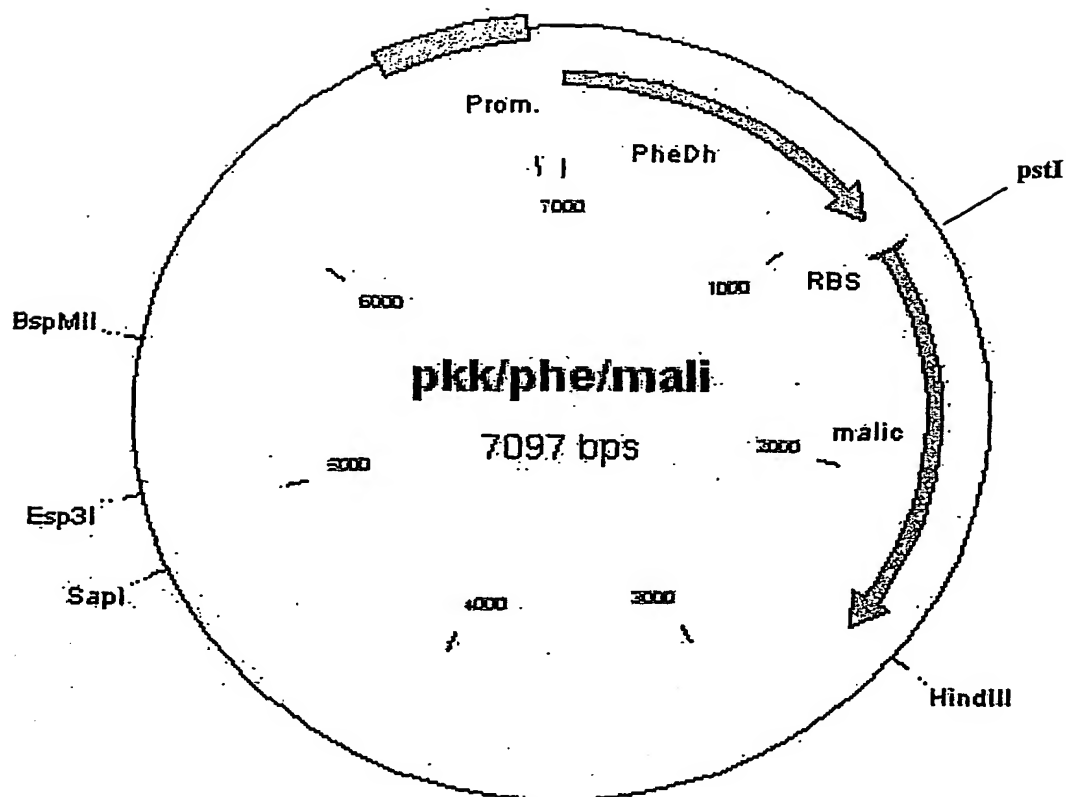
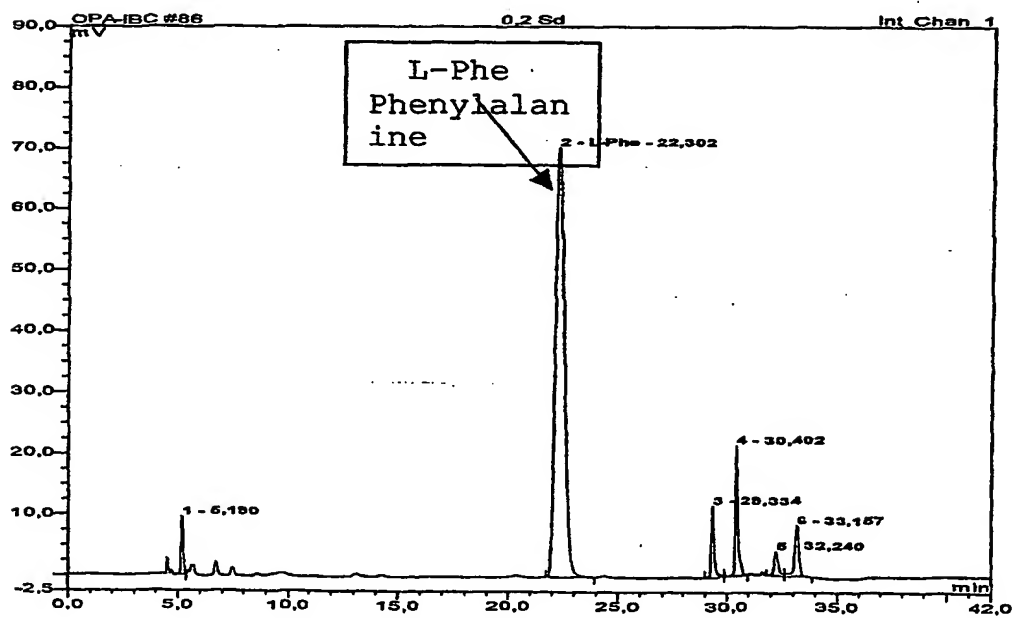


Fig. 6:



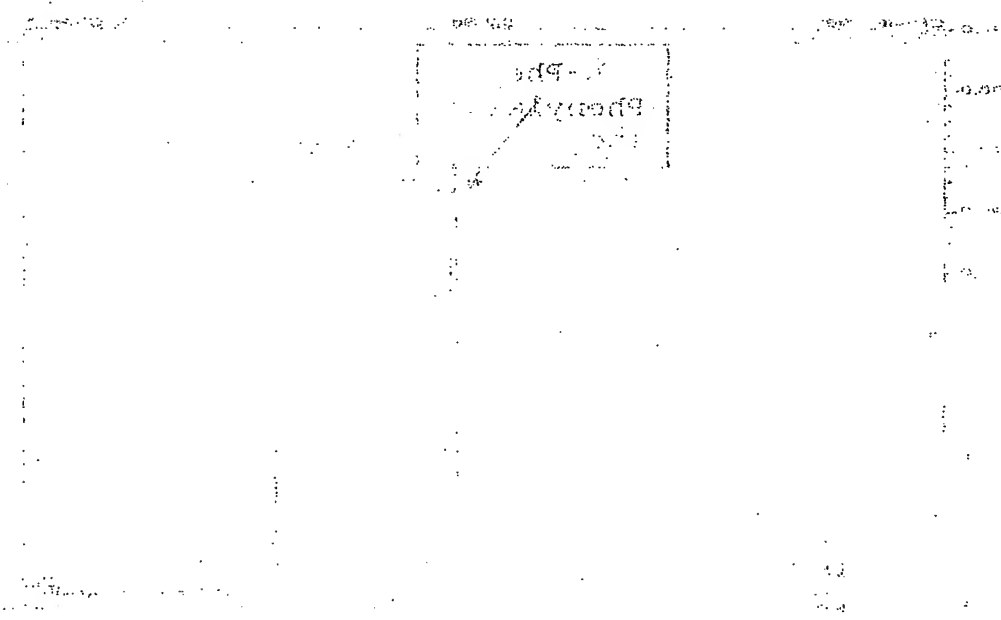
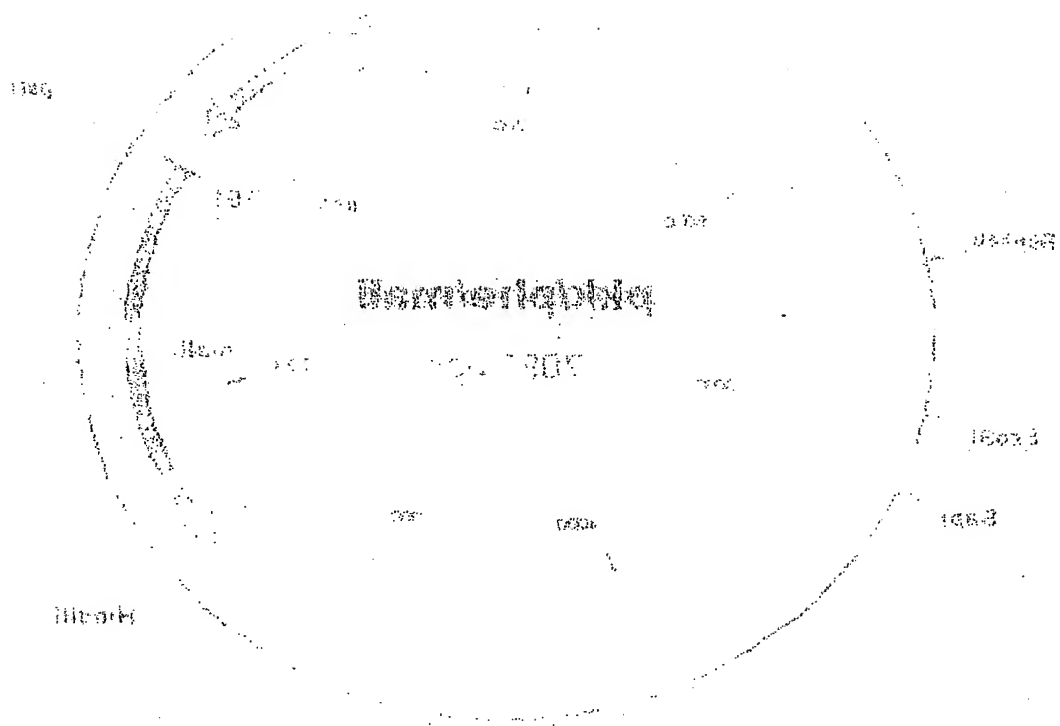


Fig. 7:

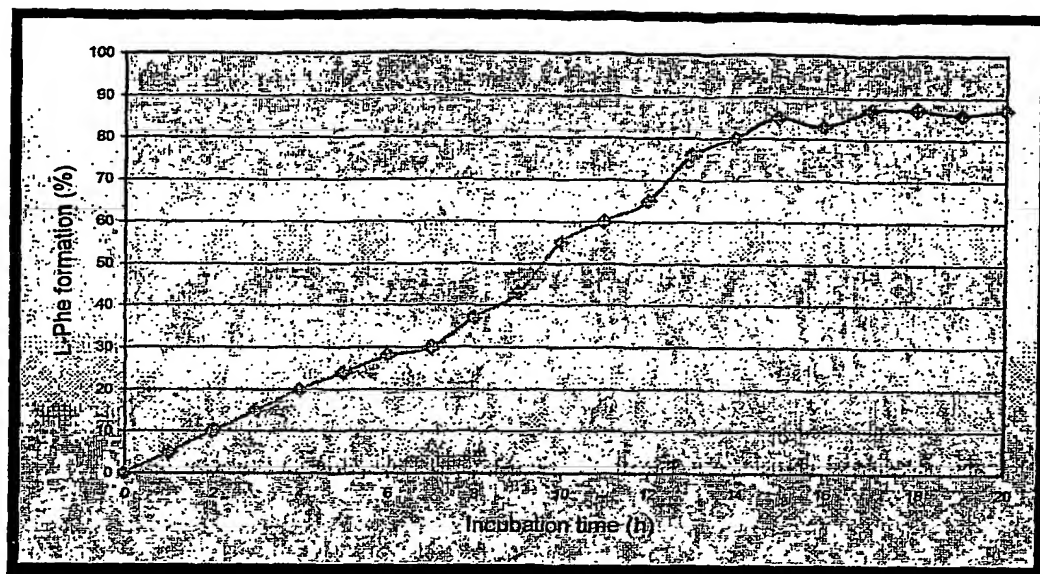
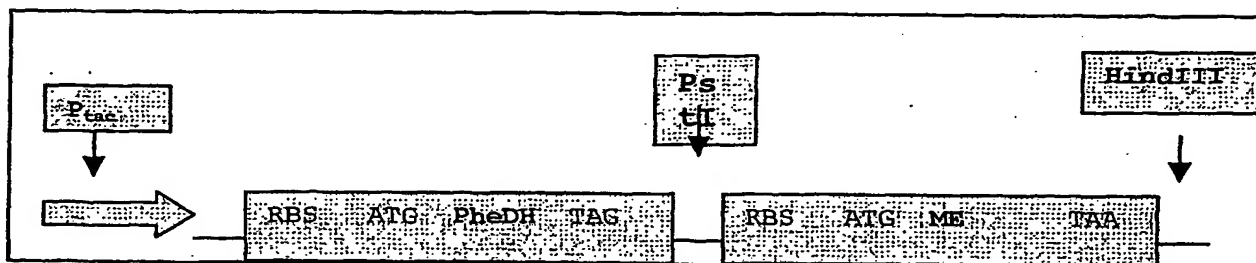


Fig. 8:



0.1

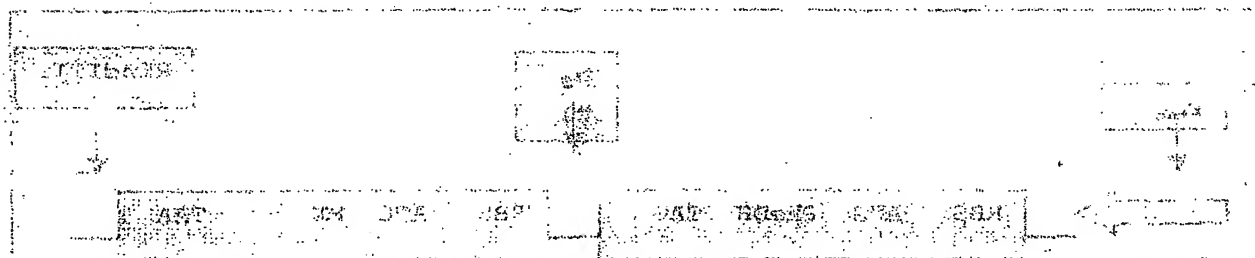
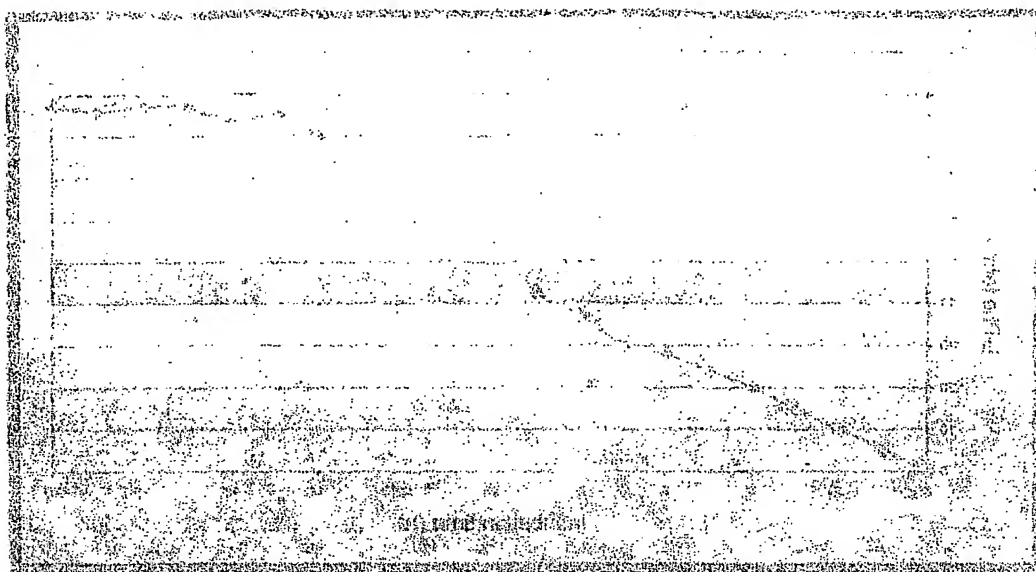


Fig. 9:

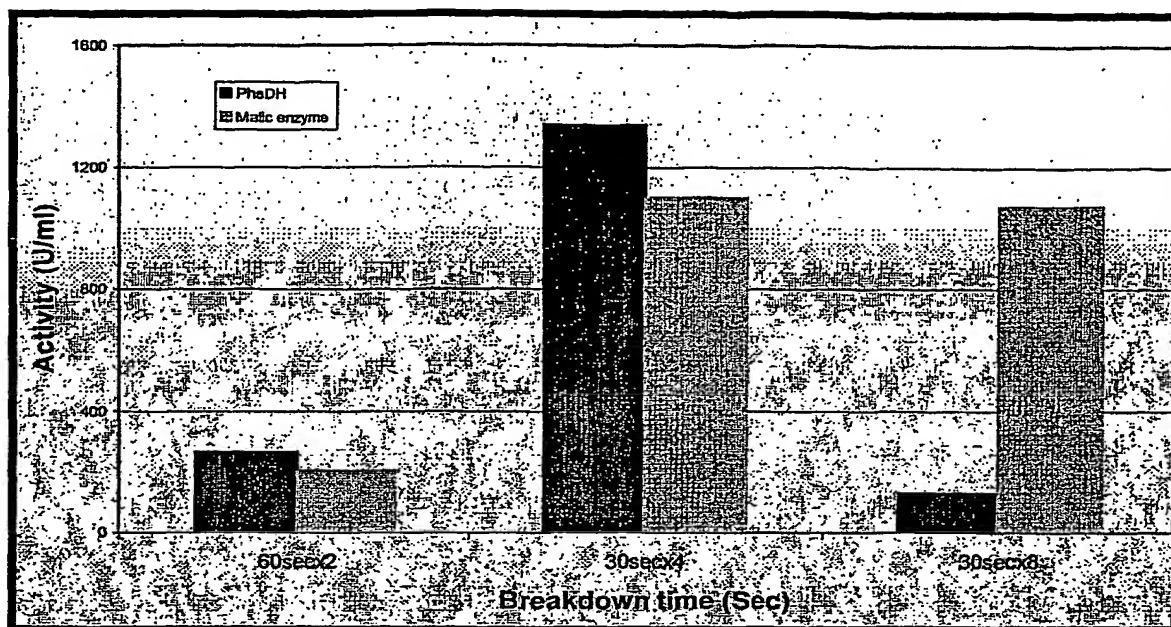
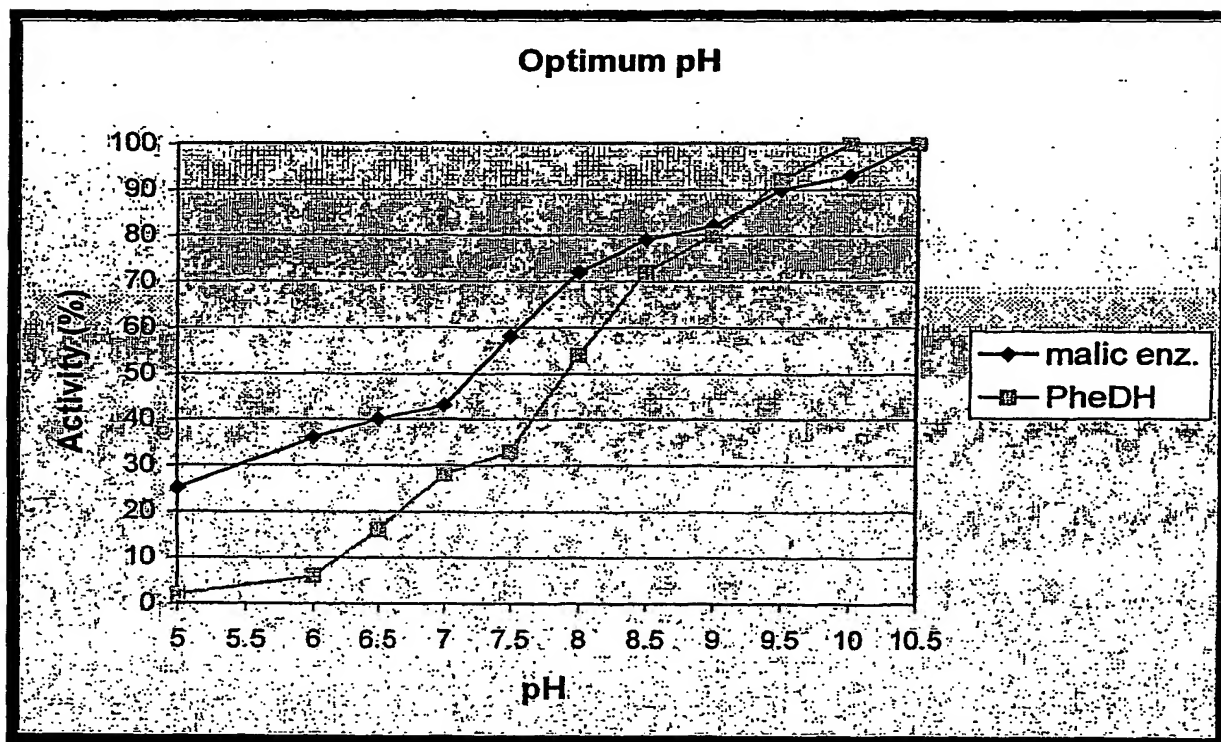
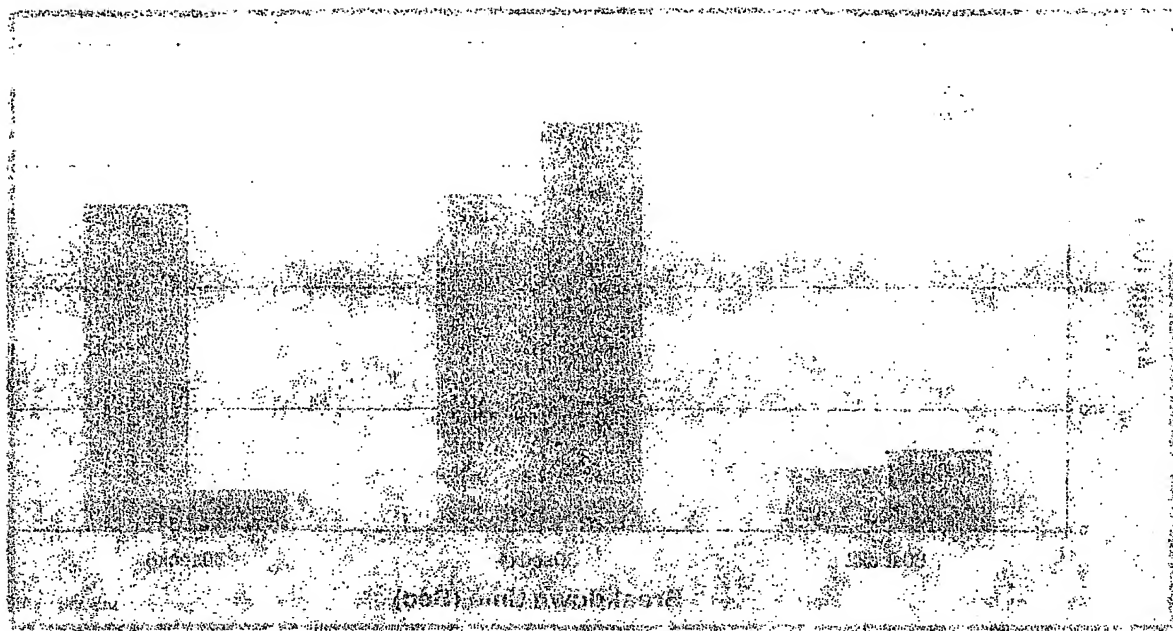


Fig. 10





01 11 11

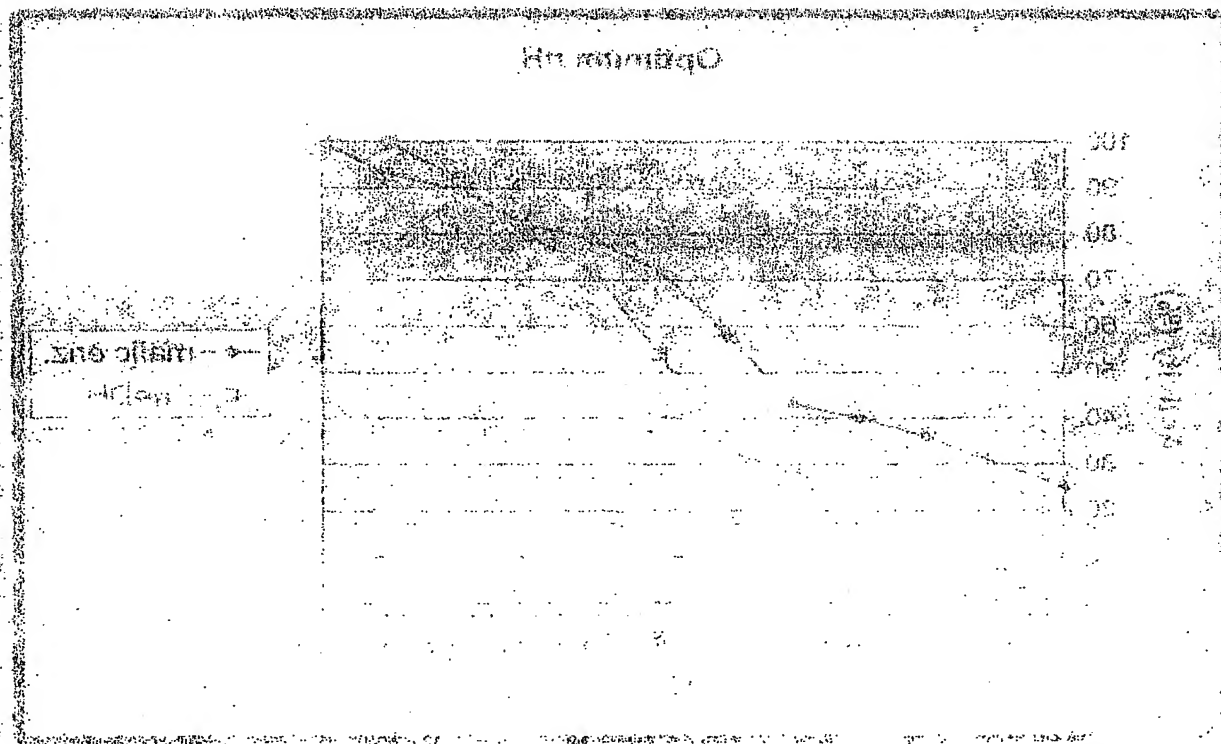


Fig. 11:

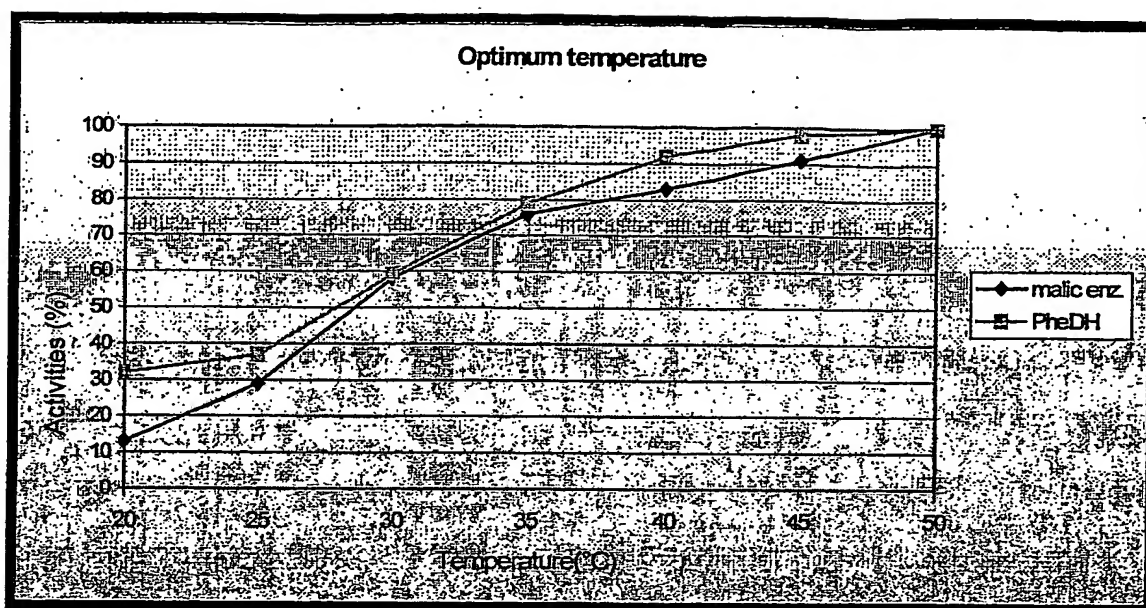
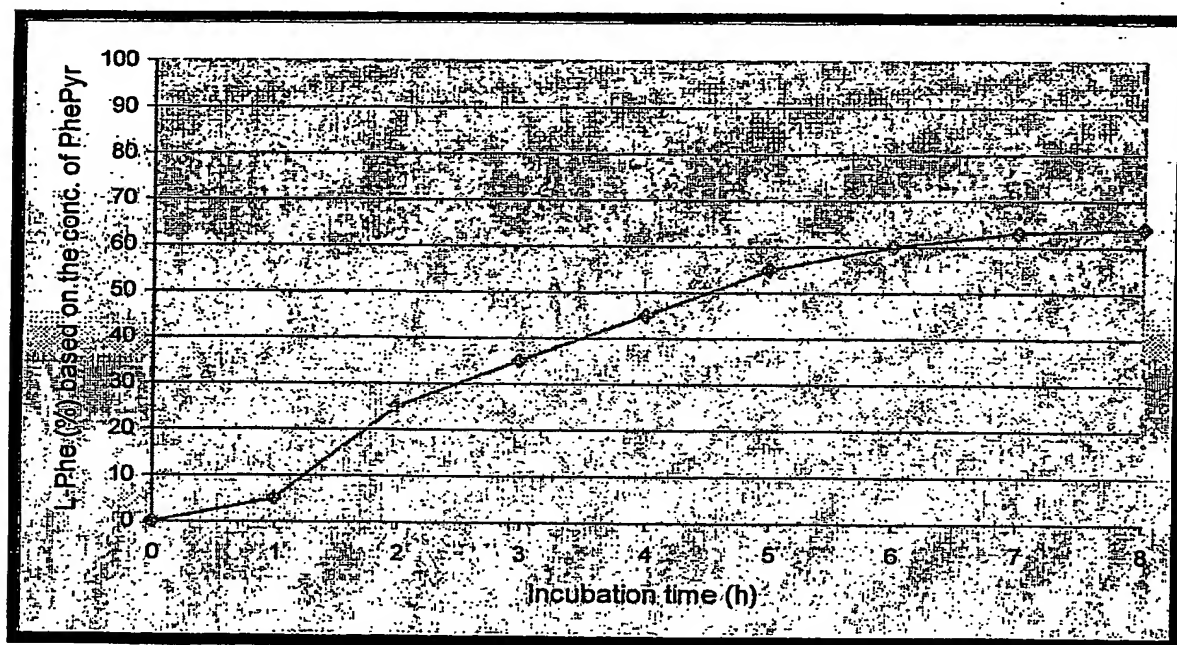


Fig. 12



## INTERNATIONAL SEARCH REPORT

Application No

PCT/EP 03/08631

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12P13/04 C12P7/04 C12P41/00 C12P13/22 C12P13/06  
C12N15/53 C12N9/04

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12P C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, WPI Data, PAJ, EMBASE, CHEM ABS Data

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99 46363 A (ARISTIDOU ARISTOS ; VALTION TEKNILLINEN (FI); RICHARD PETER (FI); T) 16 September 1999 (1999-09-16) examples 6,7,10,12-15,18,24	1-10
X	US 2002/042110 A1 (MITSUHASHI KAZUYA ET AL) 11 April 2002 (2002-04-11) paragraph '0162!	1-10
X	EP 0 385 415 A (MITSUBISHI PETROCHEMICAL CO) 5 September 1990 (1990-09-05) page 10; example 6	1-8
	-/-	



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

## \* Special categories of cited documents:

\*A\* document defining the general state of the art which is not considered to be of particular relevance

\*E\* earlier document but published on or after the international filing date

\*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

\*O\* document referring to an oral disclosure, use, exhibition or other means

\*P\* document published prior to the international filing date but later than the priority date claimed

\*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

\*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

\*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

\*&amp;\* document member of the same patent family

Date of the actual completion of the international search

12 December 2003

Date of mailing of the international search report

18/03/2004

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax (+31-70) 340-3016

Authorized officer

Lopez García, F



# INTERNATIONAL SEARCH REPORT

Application No. PCT/EP 03/08631

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>DATABASE WPI Section Ch, Week 199001 Derwent Publications Ltd., London, GB; Class B05, AN 1990-003179 XP002264717 -&amp; JP 01 285193 A (DAICEL CHEM IND LTD), 16 November 1989 (1989-11-16) page 156 abstract</p>	1-8
X	<p>DATABASE WPI Section Ch, Week 198630 Derwent Publications Ltd., London, GB; Class B05, AN 1986-194622 XP002264718 -&amp; JP 61 128895 A (TAKARA SHUZO CO LTD), 16 June 1986 (1986-06-16) abstract</p>	1-8
X	<p>GU K F ET AL: "PRODUCTION OF ESSENTIAL AMINO ACIDS IN BIOREACTORS CONTAINING ARTIFICIAL CELLS IMMOBILIZED MULTIENZYME SYSTEMS AND DEXTRAN-NAD" BIOTECHNOLOGY AND BIOENGINEERING, vol. 36, no. 3, 1990, pages 263-269, XP001156578 ISSN: 0006-3592 page 266; figures 2,7</p>	1-8
X	<p>SUYE S: "COENZYME REGENERATION WITH MALIC ENZYME REACTION SYSTEM" RECENT RESEARCH DEVELOPMENTS IN FERMENTATION AND BIOENGINEERING, XX, XX, vol. 1, no. 1, 1998, pages 55-64, XP000982890 figure 5; table 1</p>	1-8
X	<p>BOMMARIUS A S ET AL: "BIOCATALYSIS TO AMINO ACID-BASED-CHIRAL PHARMACEUTICAL-EXAMPLES AND PERSPECTIVES" JOURNAL OF MOLECULAR CATALYSIS. B, ENZYMATIC, ELSEVIER, AMSTERDAM, NL, vol. 5, no. 1998, pages 1-11, XP002929513 ISSN: 1381-1177 figure 5</p>	1-8

## INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 03/08631

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 9946363	A	16-09-1999	FI 980551 A	12-09-1999
			AU 756211 B2	09-01-2003
			AU 2730399 A	27-09-1999
			EP 0981600 A1	01-03-2000
			WO 9946363 A1	16-09-1999
			JP 2001525682 T	11-12-2001
US 2002042110	A1	11-04-2002	JP 2000236883 A	05-09-2000
			EP 1013758 A2	28-06-2000
			US 6312933 B1	06-11-2001
			US 2002127679 A1	12-09-2002
EP 0385415	A	05-09-1990	JP 2227081 A	10-09-1990
			JP 3076581 A	02-04-1991
			DE 69019843 D1	13-07-1995
			EP 0385415 A1	05-09-1990
			US 5416012 A	16-05-1995
			US 5336608 A	09-08-1994
JP 01285193	A	16-11-1989	NONE	
JP 61128895	A	16-06-1986	NONE	